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The use of induced pluripotent stem cells in drug development

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Running title

Drug discovery based on iPSC technology

Key words

iPS cell, drug development, disease modeling, control definition, animal model, personalized medicine.

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Summary

Induced pluripotent stem cell (iPSC) technology is revolutionizing medical science, allowing the exploration of disease mechanisms and novel therapeutic molecular targets, and offering opportunities of drug discovery and proof-of-concept studies in drug development. This review focuses on the recent advancements in iPSC technology including disease modeling and control setting in its analytical paradigm. We describe how iPSC technology is integrated into existing paradigms of drug development and discuss the potential of iPSC technology in personalized medicine.

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I. Introduction

The ability of cells to differentiate into various cell types—known as "pluripotency"—is a hallmark of embryonic stem cells (ESCs). Stem cells belong to one of two major categories according to their potency of differentiation: organ-specific stem cells and pluripotent stem cells. Organ-specific stem cells generally have limited potential for growth and differentiation. In contrast, pluripotent stem cells, such as ESCs[1-3] and induced pluripotent stem cells (iPSCs),[4-6] replicate in culture dishes and are theoretically capable of giving rise to any of the cell types found in the body (Figue 1).

The development of cellular reprogramming techniques leading to iPSCs has dramatically changed the landscape of stem cell research and application by providing a modality that circumvents the two major issues hampering fulfillment of the great potential of human ESCs.[4-6] One is the ethical issue associated with the derivation of human ESCs from human fertilized eggs, and the other is the immunological incompatibility between ESC-derived donor organs or cells and the recipients because of histocompatibility-antigenic factors.[4-6] As iPSCs are transforming the field of regenerative medicine, the reprogramming approach is also becoming a platform for drug discovery research.

II. Discovery of iPSCs

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II-1. Reprogramming inducers

Transduction of four genes encoding transcription factors highly functional in ESCs (i.e., Oct3/4, Sox2, Klf4, and c-Myc) was discovered to be sufficient to trigger reprogramming of both mouse and human somatic cells and to generate cells closely resembling the respective ESCs.[4-6] The term coined for these reprogrammed ESC-like cells was "iPSCs"[4]. Subsequent research from our laboratory as well as from others has revealed several alternative methods for generating iPSCs.[7-9]

Among the quartet of transcription factors involved in reprogramming[9], Oct3/4 is expressed specifically in ESCs and germ cells but not in somatic cells.[9] The forced expression of Oct3/4 in mouse or human Sox2-expressing neural stem cells can give rise to iPSCs, albeit with low reprogramming efficiency.[9] There are reports of iPSC generation even in the absence of the Oct3/4 transgene, but the efficiency of generation is very low.

Sox2 , which is a key partner of Oct3/4, is expressed almost exclusively in ESCs, germ cells, and nerve cells. The deletion of Sox2 causes the death of the embryo, suggesting its crucial role in embryogenesis.[9] Sox family proteins, including Sox2, show functional overlap with each other. Although the conventional reprogramming method requires Sox2 transgene, inhibition of the transforming growth factor beta (TGF-β) was shown to be capable of replacing Sox2 in reprogramming mouse embryonic fibroblasts.[9] Moreover, in some cell types, such as neural stem cells, melanocytes, and

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melanoma cells, Sox 2 transgene is not necessarily a requirement for iPSC generation.[9] These findings indicate the opportunistic nature of Sox transgene requirement in iPSC reprogramming.

Kruppel-like transcription factor 4 (Klf4) is a downstream target gene of the signaling pathway of the cytokine leukemia inhibitory factor—Stat3. Klf4 has overlapping functions with other Klf transcriptional factors (Klf2 and Klf5).[10] During the reprogramming process, Klf4 binds to the Oct3/4-Sox2 complex[11], and together with homeobox protein PBX1, it underpins iPSC identity by regulating expression of Nanog, one of the pluripotency-defining proteins[12]. The Klf4 transgene is not necessary for reprogramming under certain conditions such as histone deacetylase inhibition[13,14] and the absence of the tumour suppressor gene, Trp53.[15]

The reprogramming process is highly enhanced by c-Myc,[16] although its inclusion in the reprogramming process should be discouraged, given its clear oncogenic potential. c-Myc expression is ubiquitous, in contrast to the other Myc family members, N- and L-Myc.[9] L-Myc, and c-Myc mutants, all of which have little transformation activity, were shown to promote the generation of human iPSCs with more efficiently and specifically as compared with wild-type c-Myc.[7]

For these reasons, the original quartet of reprogramming factors (Oct3/4, Sox2, Klf4 and c-Myc) are not necessary under certain conditions, and could be modified accordance with the experimental context. Clearly, it is

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necessary to obtain a better understanding of the mechanisms underlying somatic cell reprogramming in order to fully validate the iPSC technology.

II-2. iPSC/ESC differentiation repertoire and tumorigenicity

In vitro culture and the differentiation of stem cells provide us with opportunities for disease modeling, drug discovery, and cell replacement therapy. The generation of specific functional cell types from ESCs/iPSCs has been demonstrated, including neural cells, vascular endothelia, smooth muscle cells, cardiomyocytes, hematopoietic cells, pancreatic insulin-producing cells, and hepatocyte-like cells.[17-23] The current differentiation repertoire includes more than 200 types of somatic cells.[24] These cells may be applied in regenerative medicine, and work is ongoing to overcome the remaining hurdles. Significant challenges in iPSC-based regenerative medicine include (i) the tumorigenic potential inherent to the reprogramming methods, (ii) the difficulty in achieving highly targeted differentiation, and (iii) the complexity of cellular transplantation techniques.[25]

Eradicating the tumorigenic potential of iPSC-derived cells is of fundamental importance to further enhance clinical transfer of the technology. Interestingly, the teratoma-forming propensities of secondary neurospheres, after transplantation into the brains of nonobese/severe combined immunodeficient mice, vary significantly depending on the origin

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of the tissue from which the iPSCs were derived.[26] For example, secondary neurospheres from iPSCs generated from adult tail-tip fibroblasts of mice showed the highest propensity for tumorigenicity, whereas those from iPSCs originating from mouse embryonic fibroblasts and gastric epithelial cells showed the lowest such propensity, the latter being comparable, in this regard, to those obtained from ESCs. Secondary neurospheres from hepatocyte iPS cells showed an intermediate teratoma-forming propensity. The use of iPSCs in regenerative medicine clearly requires further improvement of differentiation protocols in order to minimize tumorigenicity.

III. iPSC-based disease modeling

There are many potential causes for the failed translation of drug discovery from levels of molecular and animal models to human therapeutics. In particular, the success of preclinical phases of drug development is based on animal models.[27] Furthermore, <10% of the compounds that enter the clinical phase of testing reach the stage of market approval; the estimated cost of the entire drug development process is US$1.2-1.7 billion per drug.[27-29] Drug discovery/development platforms using iPSC-based disease models could be useful in filling the gap between animal models and clinical trials.

iPSC technology is expected to provide innovative tools for drug

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development via high-throughput therapeutic/toxicity screening, using differentiated cells from patient-derived iPSCs. This disease-modeling approach to drug discovery will also increase our understanding of disease progression and biology in specific cell types, which could possibly lead to redefining known aspects of diseases.[30] Patient-specific iPSCs provide not only genetic information but also potential phenotype attributes. In addition, iPSCs can be generated from patients irrespective of whether the disease is in the familial or the sporadic form. Drug screening platforms can be developed to test compounds (including biologics such as small hairpin RNAs) that are able to make the disease-related phenotype revert to that of the non-disease control.[30]

The available lines of human ESCs are variable with regard to epigenetic information, expression profile, and differentiation propensity.[31,32] Significant intrinsic variability also remains in iPSC lines, and abnormal expression of imprinted genes has been detected in a significant number of them.[33] These inter-iPSC differences were attributed to the introduction of reprogramming factors using randomly integrating viral vectors, and/or to persistent donor cell gene expression.[34] However, even if iPSCs are generated in the absence of integrating factors, intrinsic variability remains,[35-37] including in the matter of neuronal differentiation competence.[38] Moreover, expression profile analysis of integration-free human iPSCs has shown an expression signature in iPSCs that is distinct

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from those of both the original population and standard human ESCs.[35] It is also reported that there is a strong correlation between gene expression signatures and specific laboratories, in both ESC and iPSC lines, because of differences in the *in vitro* microenvironment.[39] These observations suggest that further dissecting the intrinsic variability of iPSCs may provide clues regarding the wild-type iPSCs that would be the most suitable as experimental controls and the number of control lines that should be obtained for each experiment.[35] Despite these variations, however, many lines of disease-specific iPSCs are being generated,[40] given that several studies have actually recapitulated the phenotypes of diseases in the iPSC-derived targeted cell population and that this approach now finds a place on the drug development platform as a useful tool to complement *in vivo* experiments. (Table 1). [41-46]

To avoid both inter- and intrapatient clonal variations of iPSCs, it is necessary to purify targeted cells by fluorescence-activated cell sorting or magnetic sorting using fluorescent or magnet-labeled antibodies[27] or by high-content analysis.[47,48] The control of the prominent heterogeneity of iPS-derived differentiated cells presents a technological challenge; this continues to be the major limitation of standardized high-throughput screening, although further modifications in differentiation protocols are under way in our laboratory.

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IV. iPSC-based toxicity screening

The progressive attrition of medicinal products in the long pipeline between 'hit' identification and the market has become one of the concerns of the pharmaceutical industry in the past decade.[48] The development cost of a marketable product is continuing to grow.[27-29,49] In 2001, development was abandoned because of lack of efficacy in 30% of the medicines that entered clinical trials and in another 30% because of safety concerns[49] such as cardiotoxicity and hepatotoxicity. The effective development of new drugs therefore requires predictive toxicity assays of adequate accuracy during preclinical testing. The use of human iPSCs and robust protocols to differentiate them into cardiomyocytes and hepatocytes should be able to provide straightforward assays for analyzing certain aspects of drug metabolism and for assessing probable side effects. However, technological hurdles still exist with respect to achieving the desired maturity of differentiated cells[50] and minimizing the substantial heterogeneity of iPS-derived differentiated cells for the assay. Despite these limitations, significant progress has been made.

The drug-induced blockade of the ether-a-go-go related gene 1 (hERG1) channel is reportedly associated with an increased duration of ventricular repolarization, causing prolongation of the QT interval (i.e., long QT syndrome).[51-54] Data related to the electrophysiological capacity and responsiveness of human iPSC-derived cardiomyocytes in response to

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several cardiac and noncardiac drugs have been reported.[51-54] Cardiac toxicity screening tools based on these approaches will soon become available.

The efficient generation of functional hepatocyte-like cells from iPSCs has been also reported.[20,21] The use of three-dimensional culture as well as co-culture systems (e.g., associating Kupffer and/or endothelial cells with hepatocytes in order to mimic the *in vivo* hepatic context) are among the strategies now recognized to enhance the generation of even more mature cells.[49]

To establish toxicity screening tools using iPSC technology, validation is essential. In particular, it is crucial to show high fidelity of the iPSC-based toxicity screening tools in reproducing, *in vitro,* the toxicity profiles of "hit" drugs that had been eliminated from the development pipeline because of safety concerns.

V. Challenges in iPSC-based approaches

V-1. Aging process and environmental effects

Several diseases that are characterized by onset in early life have been successfully modeled using iPSC technology.[41-46] On the other hand, in some diseases (including neurodegenerative diseases) that are age dependent, patient-specific iPSC-derived neural cells may not immediately manifest the disease phenotype as compared with normal control cells, under basal cell

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culture conditions.[30,55,56] This may also apply to drug toxicity that shows age-dependent susceptibility. Identification of disease/toxicity-related phenotypes in short-term settings *in vitro* appears particularly challenging, but it may be possible to achieve by mimicking the aging process with stressors such as oxygen reactive species, proinflammatory factors, or toxins.[30,55,56] Identification of new and more effective and relevant stressors that can accelerate the process of eliciting phenotypes in models of late-onset diseases will therefore be an important goal for future disease modeling.[30, 55,56]

Even patients with monogenetic diseases manifest large genotype–phenotype variability. Therefore, it would be more difficult to establish disease modeling from sporadic-disease iPSCs, given the complexity of the different genetic backgrounds and environmental cues involved.[27,30] It will be both challenging and exciting to examine whether the same phenotype as seen in monogenic-disease modeling could be recapitulated in sporadic-disease-iPSC-derived modeling by reproducing environmental effects *in vitro*.[27,30,55,56]

V-2. Definition of "control"

Whether in selecting a therapeutic or in toxicity assays using patient-specific iPSC-derived cells, the use of well-defined, non-disease control cells is crucial. Recent genome-wide association studies[57] have demonstrated that

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every person has disease-relevant single-nucleotide polymorphisms, and it is therefore impossible to categorically define iPSCs that represent perfect non-disease control.

Nonetheless, we think that the following two approaches are valid for deriving iPSC-positive (disease) and negative (non-disease) controls: (i) deductive and (ii) inductive. Deductive controls would include non-disease iPSC/ESC lines with modification (e.g., disease gene transgenic and disease gene knock-in), disease gene-corrected iPSC /ESC lines generated from disease iPSC/ESCs, and iPSCs with non-disease alleles from an individual patient in somatic mosaicism (Table 2). Deductive approaches define negative and positive controls in similar genetic backgrounds, providing benchmarks of disease modeling to specify differences between disease and non-disease control, whereas contributors other than the targeted gene(s) are not considered. On the other hand, inductive controls may be non-disease iPS cell lines or iPSCs from healthy individuals or from other patients (positive control). This approach could be less complicated than the deductive method, especially if noise from iPSC variations can be further reduced.

For the deductive control setting of disease modeling, the tools for achieving expression or knockout of disease genes in hiPSCs/ESCs by random integration of vectors (including viruses, bacterial artificial chromosomes, synthetic gene delivery reagents, and a

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transposon/transposase system) are useful.[58-60] Also, the current development of engineered nucleases makes targeted genome modification an attractive tool with therapeutic potential that may go beyond the development of drug screening tools.[58]

VI. iPSC-based novel drug development platform

VI-1. iPSC-based *in vitro* Phase III

Diseases can be divided into rare, monocausal genetic diseases and a large group of sporadic, multifactorial diseases. No large-scale disease modeling is currently available for the latter group. Technological advances in rapid and easy iPSC generation on a large scale will realize the possibility of both *in vitro* phase III and case-control studies by using non-disease and disease controls derived from age/gender-matched donors or from family members regardless of age/gender.[30] One of the factors facilitating the process could be to obtain a blood sample from each patient in order to generate iPSCs. iPSC generation from peripheral blood drops from each patient would allow case-control studies to be carried out, although several issues must still be resolved prior to the use of iPSCs from peripheral blood cells.[61-64] First, the differentiation potency of these iPSCs must be analyzed further.[61] Peripheral blood-derived iPSCs may preserve epigenetic memories of having been blood cells and may therefore exhibit preferred differentiation into hematopoietic lineages rather than into other

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cell types.[61,65] Another issue is how long and to what extent iPSC clones from terminally differentiated cells can be expanded.[61-64] Finally, the effect of the presence of pre-existing T-cell receptor rearrangements on the properties of iPSC or differentiated cells needs to be determined.[61,66] Besides minimizing the invasive biopsy procedures, reducing the time requirement for iPSC differentiation, resulting in lower costs, would be essential for large cohort studies, potentially leading to the discovery of novel drug targets.

VI-2. iPSC and animal model

Cell lines and animal models contribute to the exploration of disease mechanisms and drug development for various diseases. However, the animal models do not always demonstrate the same phenotypes as those seen in humans.[55] For instance, in mice the type and/or distribution of cardiac ion channels are different from those in humans, demonstrating a relatively shorter duration of action potential and higher heart rate (600 bpm).[67] An *in vitro* analysis of human cardiomyocytes is therefore critical to an understanding of the mechanism of genetic-related arrhythmias in humans.[67] Also, compounds that demonstrate significant benefit in animal models may fail to show effectiveness in clinical trials in humans.[55,68,69] The use of transgenic mice of mutant superoxide dismutase (*SOD1*), a gene found to be associated with amyotrophic lateral sclerosis,[70] enabled the identification of several compounds that relieve the disease phenotype,

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including vitamin E and creatine.[71-73] However, when these compounds were tested in humans, no clinical improvements were observed.[71-73] The toxicity of compounds is sometimes missed in cell lines and animal models because specific interactions with human biological processes cannot be recapitulated in these systems.[27] Also, the use of animal models for toxicity assays may be ethically problematic, the animals may be expensive to purchase and maintain, and the process may be difficult to automate.[27] Clearly, we require different drug screening models that complement these systems and represent the human condition with high fidelity.[74] iPSCs are expected to fulfill these requirement and are amenable to the demands of drug development. There are nonetheless great advantages associated with cell line-based models (which could be used for homologous culture, yielding reproducible results) and for animal models (which provide information regarding optimal time window, drug delivery, metabolism, etc.) (Figure 2). Integrated drug screening systems, consisting of disease-specific iPSC-based models as well as cell lines and animal models, would greatly enhance the efficiency of translational drug research.

VI-3. Personalized medicine

The striking advantage of using iPSCs rather than ESC-based approaches is that iPSCs can be derived from any individual with relative ease, thereby allowing development of a personalized study platform on

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individual genomic information. iPSCs and differentiated cells from the iPSCs retain their personal identity, like an alter ego, suggesting that iPSC technology can be applied to disease-, patient-, and finally person-specific approaches to examine the individual differences in pharmacokinetic/pharmacodynamic features (Figure 3). Given that everyone will almost certainly become a patient at least once in his or her lifetime, individual iPSC-based predictive therapeutic and toxicity profiling of all drugs available in multiple cell types will be a logical and attractive approach. This "pharmaco-iPSCellomic" analysis (Figure 4) could eventually be available in an array-based format for high-throughput assay before specific drug therapy is prescribed for a certain disease condition.

VII. Conclusion

The potential of the iPS cell technology in drug discovery is enormous.[75] At the same time, the technology is still in its infancy with numerous challenges to overcome before its clinical translation is complete. The long journey has just begun. It may take years to reach the eventual goals, but the iPSC technology itself, combined with existing methods and models, will begin to contribute to the development of new cures.

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Conflict of Interest

The authors declared no conflict of interest.

Legend

Table 1. Disease modeling using disease-specific iPSCs.

Table 2. Proposed definition of “control” in induced pluripotent stem cell research.

Figure 1. Differentiation potential of iPSC.

Figure 2. Combined approach of animal models and iPSC technology.

Figure 3. Personalized medicine based on iPSC technology.

Figure 4. “Phamaco-iPSCellomics” by person-specific iPSCs.

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Figure 1

Generation of induced pluripotent stem cells (iPSCs) and their differentiation potential. iPSCs are derived from easily accessible somatic cells. In contrast to organ-specific stem cells, pluripotent stem cells such as embryonic stem cells and iPSCs show the ability to differentiate into many different cell types in culture. This allows *in vitro* generation of specific tissue cell types with the characteristics of the disease phenotype, from patient-derived iPSCs.

Figure 2

Combined approach involving animal models and induced pluripotent stem cell (iPSC) technology. The new iPSC technology is complemented by a drug development strategy in preclinical settings that uses animal models and other conventional approaches.

Figure 3

Personalized medicine based on induced pluripotent stem cell (iPSC) technology. iPSC technology is highly amenable to individualized approaches. Person-specific iPSCs can be derived, differentiated into specific cell types, and used for therapeutic/toxicity response assays.

Figure 4

”Phamaco-iPSCellomics” by person-specific iPSCs. iPSCs derived from individual subjects/patients can be differentiated into multiple cell types, thereby providing a personalized iPS-cellome platform. This cell-based system can be used for drug discovery and selection of clinical therapeutics with various biomarker end points.

Figure 1

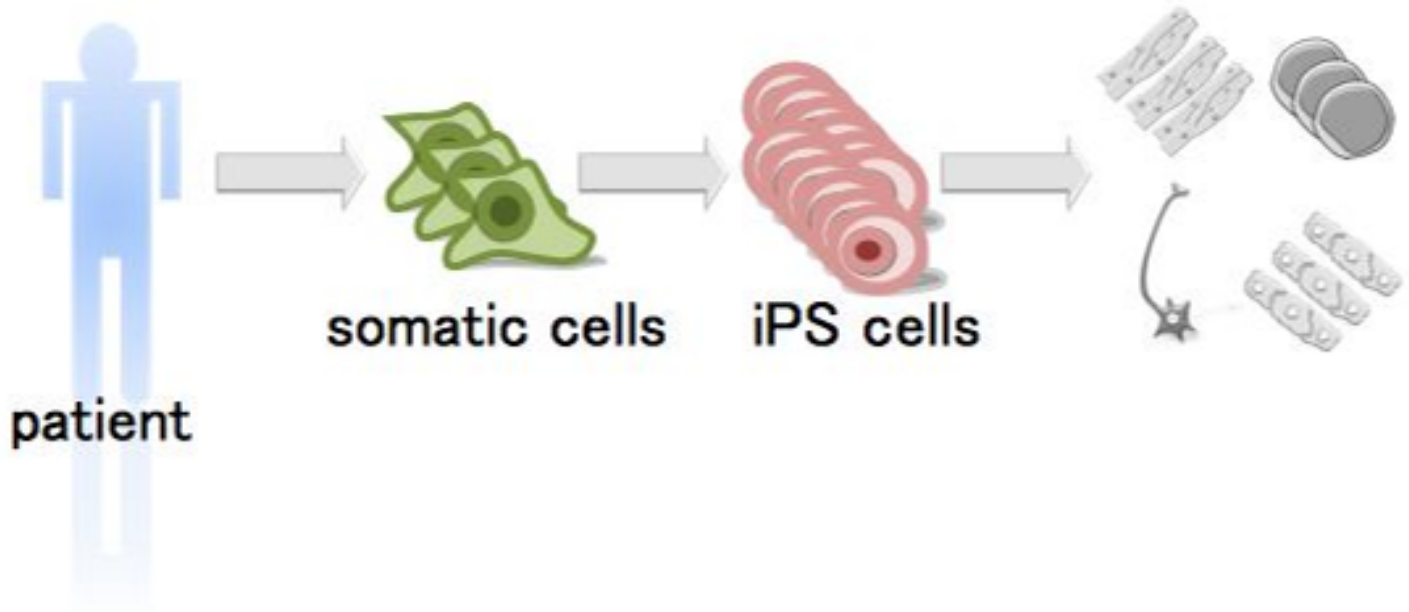

Figure 2

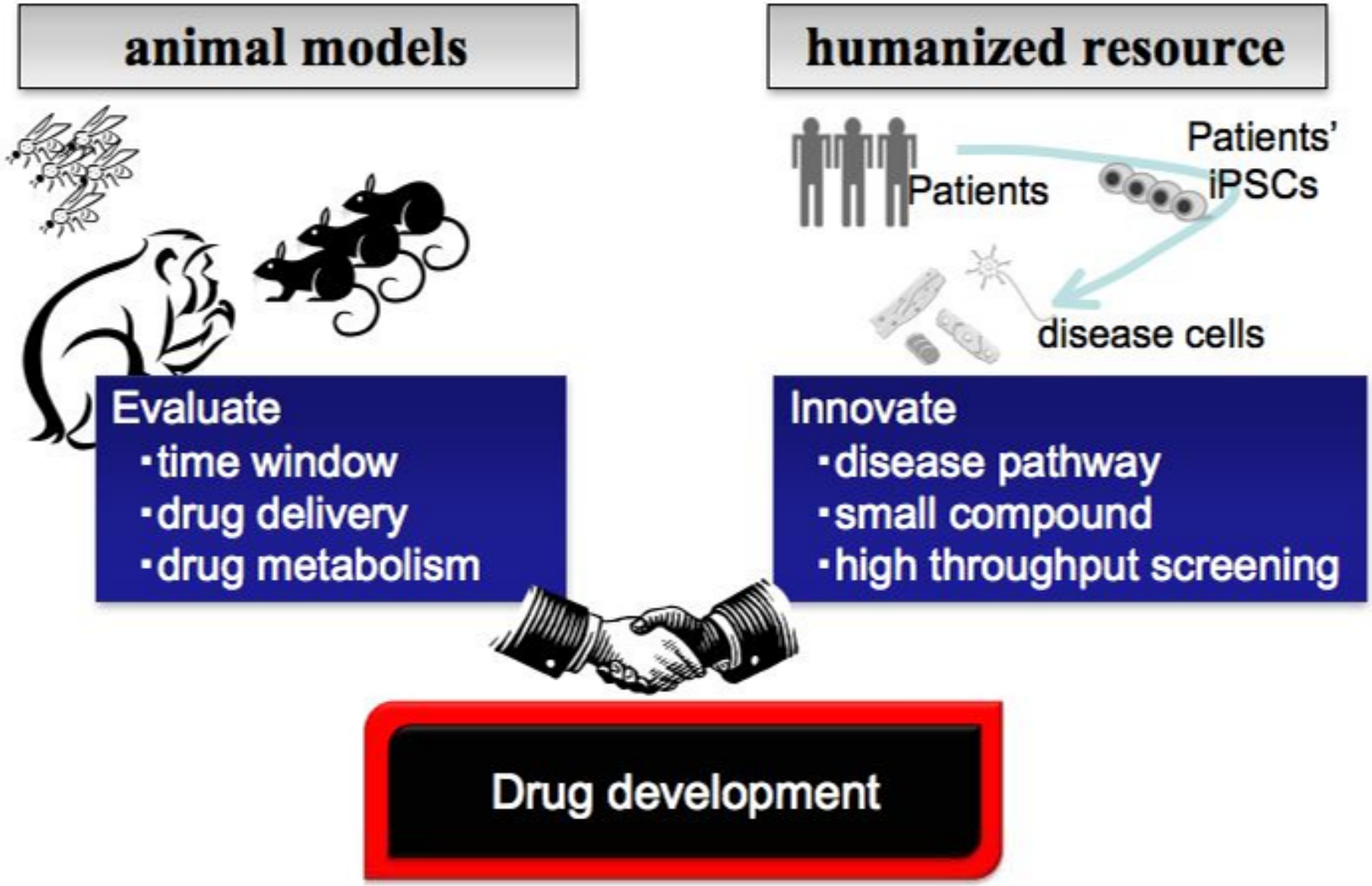
animal models
humanized resource
Patients
Patients'
iPSCs
disease cells
Evaluate
・time window
・drug delivery
・drug metabolism
Innovate
・disease pathway
・small compound
・high throughput screening
Drug development

Figure 3

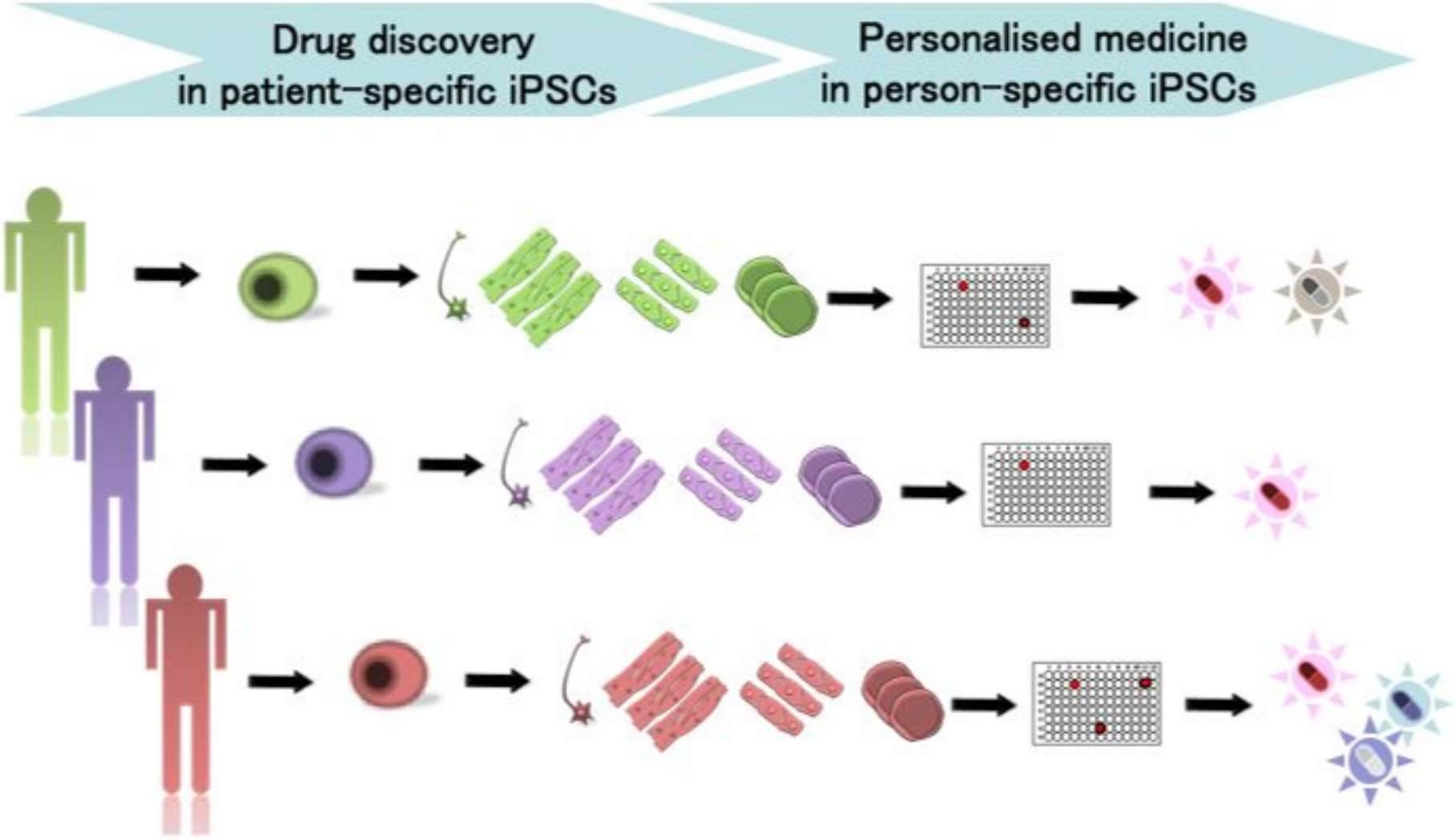
Drug discovery
in patient-specific iPSCs
Personalised medicine
in person-specific iPSCs

Figure 4

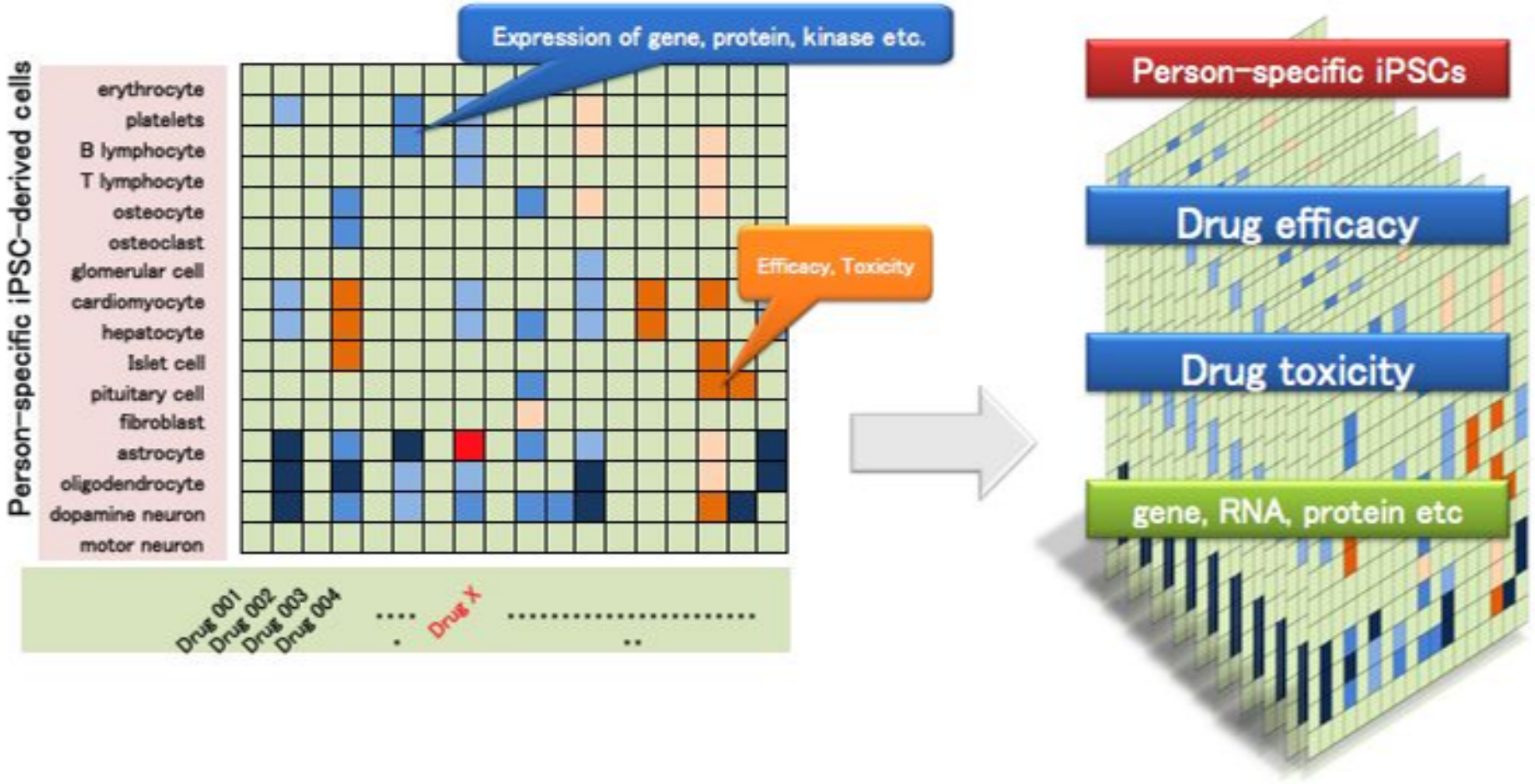
Person-specific iPSC-derived cells
erythrocyte
platelets
B lymphocyte
T lymphocyte
osteocyte
osteoclast
glomerular cell
cardiomyocyte
hepatocyte
Islet cell
pituitary cell
fibroblast
astrocyte
oligodendrocyte
dopamine neuron
motor neuron
Expression of gene, protein, kinase etc.
Efficacy, Toxicity
Drug 001
Drug 002
Drug 003
Drug 004
Drug X
Person-specific iPSCs
Drug efficacy
Drug toxicity
gene, RNA, protein etc

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Table 1 Disease modeling using disease-specific iPSCs.

Disease (responsible gene)	Inheritance pattern	Age of onset	Recapitulated phenotype / proof of drug efficacy
Spinal muscular atrophy[41] (SMN1, SMN2)	AR	Infancy to Adolescence	1) Decreased no. (%) of $ChAT^{+}$/$Tuj1^{+}$ neurons. 2) Decreased SMN protein level (evaluated with WB/IA). 3) Rescue phenotype 2 with 1mmol/l Valproic acid.
Familial dysautonomia[42] (IKBKAP)	AR	Infancy	1) Increased abnormal splicing in differentiated neural crest. 2) Decreased no. (%) of ASCL1+, Tuj1+ neurons. 3) Migratory dysfunction (scratch assay). 4) Partial Rescue phenotype 1, 2 with 100 μmol/l kinetin.
Fanconi anemia[43] (FANC A~N)	AR /XR	First decade ~ 4/5th decade	1) Unsuccessful at obtain iPSCs from patient's fibroblast →after '*in vitro* genetic correction' of patient's fibroblast, Successful in obtaining iPSCs (chromosomal instability). 2) Differentiate into CD34+/hematopoietic progenitors.
Dyskeratosis congenita[44] (XR: DKC1)	XR (AR/AD)	Adolescence	1) Elongated telomere in iPSCs (TERT/TERC ↑). 2) Shortened telomere after differentiation (TERT/TERC ↓).
LEOPARD syndrome[45] (PTPN11, RAF1, SHOC2)	AD	Infancy to Adolescence	1) Enlarged cell size of differentiated cardiomyocyte. 2) Inactivated RAS-MAPK pathway (bFGF induction).
Rett syndrome[46] (MeCP2)	XR	6-18 months	1) Reduced no. of glutamatergic synapse and morphological alterations (synapsin puncta at dendrites), rescued by IGF-1 (ng/ml). 2) Reduced RTT protein level/cell size, and rescue by gentamicin (100 μg/ml) at Q244X clone. 3) Reduced activity-dependent calcium transients. 4) Reduced spontaneous postsynaptic currents.

AD: autosomal dominant, AR: autosomal recessive, IA: immunological analysis, IGF-1: insulin-like growth factor 1, iPSC: induced pluripotent stem cell, TERC: telomerase RNA component, TERT: telomerase reverse transcriptase gene, WB: western blot analysis, XR: X-linked recessive.

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Table 2 proposed definition of "control" in induced pluripotent stem cell research

Deductive approach

Embryonic stem cell line with and without disease-introducing genetic modification

Non-disease induced pluripotent stem (iPS) cell line with and without disease-introducing genetic modification

Disease iPS cell with and without disease-correcting genetic modification

iPS cell from somatic mosaic with and without disease allele

Inductive approach

iPS cell from a patient and a disease-free family member

Disease genetic risk-ascertained iPS cell lines (preferably as a risk-absent non-disease control)

iPS cell lines from disease-phenotyped individuals (healthy or disease control)